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Identification of the Minimal Replicon of *Lactococcus lactis* subsp. *lactis* UC317 Plasmid pCI305

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Replication functions of the stable, cryptic 8.7-kilobase (kb) plasmid pCI305 from multi-plasmid-containing *Lactococcus lactis* subsp. *lactis* UC317 were studied. Analysis of this replicon was facilitated by the construction of replication probe vectors that consisted of the pBR322 replication region, a pUC18-derived multiple cloning site, and either the *cat* gene of pC194 (pCI341; 3.1 kb) or the *erm* gene of pAMβ1 (pCI3330; 4.0 kb). Plasmid pCI305 was introduced into plasmid-free *L. lactis* subsp. *lactis* MG1363Sm, a streptomycin-resistant derivative of MG1363, by a transformation procedure with the 75-kb lactose-proteinase plasmid pCI301 of UC317 as a marker plasmid. A combination of transposon Tn*5* mutagenesis and subcloning in pCI341 and pCI3330 with individual Tn*5* insertions around the replication region facilitated the identification of a 1.6-kb minimal replicon on pCI305. This region was separable into two domains: (i) a 1.3-kb region (*repB*) encoding a *trans*-acting function (in vitro transcription-translation studies suggested the involvement of a 48-kilodalton protein); and (ii) a 0.3-kb region (*repA*) sufficient to direct replication when provided with *repB* in *trans* and thus probably containing the origin of replication. Lactococcus-*Escherichia coli* shuttle vectors based on the pCI305 replication region were constructed.

The lactococci are important in dairy and other food fermentations (9) and are characterized by an abundance of plasmid DNA (34). Although some industrially significant traits, including lactose assimilation (Lac phenotype), proteinase activity (Prt phenotype), citrate utilization, and bacteriophage insensitivity, can consistently be linked to plasmid DNA (18) and have consequently been the subject of intensive studies (12), the majority of plasmids remain phenotypically cryptic.

Although the small, cryptic plasmids pWV01 (2.25 kilobases [kb]) from *Lactococcus lactis* subsp. *cremoris* Wg2 (38) and pSH71 (2.06 kb) from *L. lactis* subsp. *lactis* 712 (15) have been used in the construction of cloning, expression signal probe, positive selection, and expression vectors (12, 13, 19, 25, 45, 46, 49), lactococcal plasmid replication, partition, and stability functions have not been extensively studied. Only pSH71, the nucleotide sequence of which has been determined (12), has been analyzed in detail. Four potential open reading frames exist on pSH71, the largest of which (*repA*) specifies a *trans*-acting, DNA-binding replication protein (13). The origin of replication on this plasmid has been localized to a 0.6-kb A+T-rich segment that harbors a series of direct repeat structures (13). Significantly, the related pWV01 and pSH71 replicons (12) were characterized by extensive host ranges, sustaining replication in *Bacillus*, *Staphylococcus aureus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Propionibacterium* species and some *Escherichia coli* strains, in addition to lactococci (8, 12, 16, 25, 27, 50).

The characterization of an 8.7-kb stable cryptic plasmid, pCI305, from *L. lactis* subsp. *lactis* UC317 is reported in this study. The identification of a 1.6-kb minimal replicon was achieved by a combination of site-directed Tn*5* mutagenesis allied to subcloning in replication probe vectors. This region was separable into *trans* (*repB*)- and *cis* (*repA*)-acting segments. Evidence is provided implicating a 48-kilodalton (kDa) protein, produced by *repB*, in pCI305 replication.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media. *L. lactis* subsp. *lactis* strains were grown in M17 medium (44) containing lactose or glucose (GM17). *E. coli* and *Bacillus subtilis* were subcultured in Luria-Bertani medium (32). Chloramphenicol (5, 20, and 10 μg/ml for *L. lactis* subsp. *lactis*, *E. coli*, and *B. subtilis*, respectively), erythromycin (1 and 300 μg/ml for *L. lactis* subsp. *lactis* and *E. coli*, respectively), and ampicillin (100 μg/ml), tetracycline (10 μg/ml) and kanamycin (50 μg/ml) for *E. coli* were added to media when required.

Plasmid preparation, analysis, and modifications. Rapid preparation of plasmid DNA from lactococcal strains was by the method of Anderson and McKay (1). Plasmid profiles were analyzed as described in the accompanying report (22). Purified plasmid DNA was isolated from lactococci as described by Anderson and McKay (1) and was further treated as outlined elsewhere (22). Preparative amounts of plasmid DNA were isolated from *E. coli* as outlined by Birnboim and Doly (4) followed by CsCl-ethidium bromide density gradient ultracentrifugation. A scaled-down version of this procedure was also used in rapid screening and restriction analysis of DNA from *E. coli* clones, except that the lysozyme treatment and phenol-chloroform and chloroform extraction steps were omitted. Restriction endonuclease digestions of plasmid DNA were analyzed as described elsewhere (22). Restriction endonucleases, calf intestinal alkaline phosphatase, Klenow fragment of DNA polymerase I, and T4 DNA ligase were supplied by the Boehringer Corporation Ltd., Dublin, Ireland, and were used as recommended by the manufacturer. Recovery of DNA fragments from agarose gels was achieved by electrophoresis onto dialysis membranes (32). Unless otherwise stated, all cloning experiments were conducted in *E. coli*.

Transformation. *E. coli* was transformed by the method of Mandel and Higa (31). Polyethylene glycol-induced transformation of *L. lactis* subsp. *lactis* protoplasts was performed as described by Gasson and Anderson (16). Lac^+ transfor-

TABLE 1. Bacterial strains, bacteriophage, and plasmids

Strain, phage, or plasmid	Characteristics	Reference or source
Lactococcus lactis subsp. *lactis*		
UC317	Wild-type strain, Lac$^+$ Prt$^+$	22
MG1363Sm	Plasmid-free derivative of *L. lactis* subsp. *lactis* 712, Sm^r	15
FH054	MG1363Sm containing pCI301 and pCI305	This work
FH052	FH054 cured of pCI301	This work
Escherichia coli		
HB101	F$^-$ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-	6
LE392	F$^-$ *hsdr514* (r_K^- m_K^+) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ^-	32
JM83	*ara*Δ*lacpro strA thi* Δ*80dlacZ* Δ*M15*	47
V517	Source of size reference plasmid molecules	29
Bacillus subtilis		
PSL-1	*arg leu thi* r$^-$ m$^-$ *stp recE4*	37
Bacteriophage λ467	λ*b*221 *rex*::Tn*5* *c*I857 *O*am29 *P*am80	10
Plasmids		
pBR322	4.3-kb *E. coli* cloning vector, Ap^r Tc^r	5
pUC18	2.7-kb *E. coli* positive selection vector, Ap^r *lacZ*	47
pIC20R	2.7-kb *E. coli* positive selection vector, Ap^r *lacZ*	33
pCK1	5.5-kb *E. coli*-lactococcus shuttle vector, Km^r Cm^r	16
pIL204	5.5-kb lactococcus vector, Em^r	40
pCI301	75-kb plasmid from UC317, Lac$^+$ Prt$^+$	22
pCI305	Cryptic 8.7-kb plasmid from UC317	22
pCI341	3.1-kb replication probe vector, Cm^r	This work
pCI3330	4.0-kb replication probe vector, Em^r	This work
pCI350, pCI351	pCI305 cloned as a single *Xba*I fragment in both orientations in pCI341, Cm^r	This work
pCI3301 through pCI3320	pCI350::Tn*5* derivatives, Km^r Cm^r	This work
pCI369	2.6-kb *Xba*I-*Bgl*II fragment of pCI350 subcloned in *Xba*I-*Bam*HI-cleaved pCI341, Cm^r	This work
pCI374	1.8-kb *Hpa*I-*Xba*I fragment of pCI3303 subcloned in *Hin*dII-*Xba*I-cleaved pCI341, Cm^r	This work
pCI375	2.0-kb *Hpa*I-*Bgl*II fragment of pCI3307 subcloned in *Hin*dII-*Bam*HI-cleaved pCI341, Cm^r	This work
pCI376	2.7-kb *Hpa*I-*Bgl*II fragment of pCI3317 subcloned in *Hin*dII-*Bam*HI-cleaved pCI341, Cm^r	This work
pCI378	4.2-kb *Hin*dII-*Pvu*II fragment of pCI369 religated, Cm^r	This work
pCI379	1.4-kb *Hpa*I-*Bgl*II fragment of pCI3305 subcloned in *Hin*dII-*Bam*HI-cleaved pCI341, Cm^r	This work
pCI380	1.5-kb *Hpa*I-*Xba*I fragment of pCI3305 subcloned in *Hin*dII-*Xba*I-cleaved pCI341, Cm^r	This work
pCI3331	2.6-kb *Xba*I-*Bgl*II fragment of pCI350 subcloned in *Xba*I-*Bam*HI-cleaved pCI3330, Em^r	This work

mants were selected on M17 agar containing 1% lactose with 0.5 M sucrose as the osmotic stabilizer and 0.004% bromocresol purple as the pH indicator. Lac$^+$ transformants produced yellow colonies on this medium, whereas Lac$^-$ colonies were white. Chloramphenicol-resistant (Cm^r) and/or erythromycin-resistant (Em^r) transformants were selected on GM17 agar containing 0.5 M sucrose and 5 μg of chloramphenicol per ml and/or 1 μg of erythromycin per ml. Protoplast transformation of *B. subtilis* was by the protocol of Chang and Cohen (7).

Tn*5* mutagenesis. Preparation of high-titer λ467 lysates by propagation on *E. coli* LE392 and Tn*5* mutagenesis were performed as described by de Bruijn and Lupski (10). Tn*5* insertions were mapped by correlating the results of at least five single, double, and triple restriction digests per insertion.

In vitro transcription-translation. DNA-directed in vitro translation studies were used to identify protein products from within the pCI305 minimal replicon. ^{35}S-labeled proteins were electrophoresed on 0.1% sodium dodecyl sulfate (SDS)–15% polyacrylamide gels (26). Gels were fixed in 7% acetic acid, impregnated with Amplify, dried, and exposed to Hyperfilm-MP autoradiography film at −80°C for 48 to 96 h with an intensifying screen. ^{14}C-methylated proteins were used as molecular weight markers. The procaryotic DNA-directed translation kit and other reagents were obtained from Amersham International, Buckinghamshire, England.

Plasmid stability studies. Single-colony isolates of relevant plasmid-carrying *L. lactis* subsp. *lactis* strains grown on the appropriate antibiotic were inoculated into GM17 broth and subcultured for approximately 50 and 100 generations in the absence of selective pressure. Cultures were spread plated on GM17 agar, and 200 single colonies were tested for retention of the Cm^r or Em^r marker.

Preparation of DNA probes and hybridization analysis. DNA probes were prepared with biotin-11-dUTP according to specifications supplied with the BRL (Paisley, Scotland) nick translation kit (8160 SB) and detected using the BRL DNA detection system (8239 SA). DNA was transferred from agarose gels to nitrocellulose filters by the method of

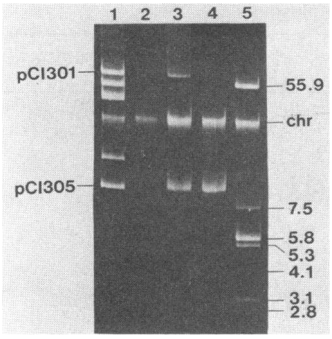

FIG. 1. Plasmid profiles of *Lactococcus lactis* subsp. *lactis* UC317 (lane 1), MG1363Sm (lane 2), FH054 (lane 3), and FH052 (lane 4) and of *E. coli* V517 (lane 5). The positions of the 75-kb Lac Prt plasmid, pCI301, of UC317, used as a marker plasmid in transformation experiments, and the 8.7-kb cryptic plasmid pCI305 are indicated. Numbers on the right refer to sizes (kilobases) of marker plasmids in V517. chr, Chromosomal DNA.

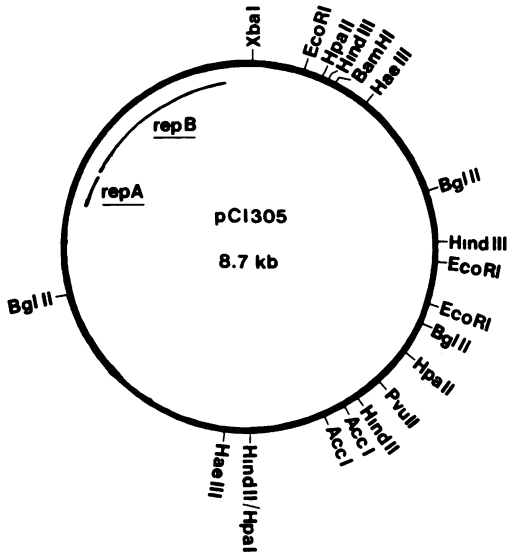

FIG. 2. Restriction map of pCI305. The unique *Xba*I site was chosen to map at position zero. The positions of replication control loci, *repA* and *repB*, are indicated (see the text for details).

Southern (42) as modified by Wahl et al. (51). Hybridization was allowed to proceed for 12 h at 65°C in 5× SSC (1× SSC is 0.15 M NaCl–0.015 M trisodium citrate)–0.1% polyvinylpyrrolidone–0.1% Ficoll–0.1% bovine serum albumin–0.1% SDS–100 μg of denatured herring sperm DNA per ml. Posthybridization washes consisted of four 15-min washes in 2× SSC–0.1% SDS at 65°C, two 15-min washes in 0.2× SSC–0.1% SDS at 65°C, and one 15-min wash in 0.1× SSC–0.1% SDS at 65°C followed by two final washes in 0.1× SSC–0.1% SDS at room temperature.

RESULTS

Identification and physical characterization of pCI305. *L. lactis* subsp. *lactis* UC317 harbors five plasmids (Fig. 1, lane 1), the largest of which, pCI301 (75 kb), encodes Lac Prt (22). The smallest cryptic plasmid in UC317, pCI305 (8.7 kb), was particularly stable under conditions such as protoplasting and regeneration or growth in the presence of plasmid-curing agents, which successfully cured all other plasmids from this strain. Because of this stability and because of its relatively small size, pCI305 was selected for the analysis of *L. lactis* subsp. *lactis* plasmid replication functions.

pCI305 was introduced into plasmid-free *L. lactis* subsp. *lactis* MG1363Sm (Fig. 1, lane 2) by transformation with total plasmid DNA from UC317, selecting for Lac^+ transformants (1.2×10^2 per μg of input DNA). Lac^+ isolates, which were also Prt^+, contained pCI301 and were screened for cotransformation of cryptic plasmids; 46.6% of Lac^+ Prt^+ transformants also contained pCI305 (Fig. 1, lane 3). A spontaneous Lac^- Prt^- derivative of one such transformant was cured of pCI301 but retained pCI305 (Fig. 1, lane 4).

A restriction map of pCI305 was constructed (Fig. 2). The plasmid possessed single sites for *Bam*HI, *Hpa*I, *Pvu*II, and *Xba*I. No sites were present for *Ava*I, *Bst*EII, *Eco*RV, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Stu*I, and *Xho*I.

Construction of replication probe vectors. Replication probe plasmids were constructed by the following procedure. The larger *Eco*RI-*Pvu*II fragments of both pBR322, which contains the replication functions and the ampicillin resistance (Ap^r) determinant (5), and pCK1, which contains the kanamycin resistance (Km^r) and Cm^r markers derived from pUB110 and pC194, respectively, and the pSH71 replication genes (16) were purified, ligated, and transformed into *E. coli* HB101, selecting for Ap^r Km^r Cm^r. A representative isolate contained a 6.5-kb plasmid, pCI336, which exhibited the correct restriction patterns. Replacement of the larger *Eco*RI-*Pst*I fragment of pCI336 with a 39-base-pair *Eco*RI-*Pst*I fragment of pUC18 (47) resulted in the simultaneous introduction of a multiple-cloning site and the elimination of the Ap^r gene of pCI336. The resulting plasmid, pCI339 (6.0 kb), was deleted completely of the lactococcal replication genes and of the Km^r determinant by purifying, end filling, and ligating the larger *Eco*RI-*Bgl*II fragment and selecting for Cm^r Km^s transformants of HB101. The *Eco*RI site was recreated in this procedure. This resulted in the replication probe vector pCI341 (3.1 kb; Fig. 3), which was incapable of transforming MG1363Sm to Cm^r, confirming that pBR322 replication genes are nonfunctional in *L. lactis* subsp. *lactis*.

A second vector in which the Cm^r marker was replaced by a pAMβ1-derived Em^r gene was constructed by inserting a 2.5-kb *Eco*RI-*Kpn*I fragment of pIL204 (40) between the *Eco*RI and *Kpn*I sites of pCI341, generating pCI373 (5.6 kb), which contains both Cm^r and Em^r markers. The Cm^r marker was deleted from pCI373 by purifying, end filling, and ligating the larger *Eco*RI-*Pvu*II fragment of this plasmid and selecting for Em^r Cm^s transformants of HB101. This yielded pCI3330 (4.0 kb; Fig. 3).

Both the *cat* and *erm* determinants of pCI341 and pCI3330, respectively, are efficiently expressed in *E. coli* (21, 30) and in *L. lactis* subsp. *lactis* (17, 25), in addition to species of a wide range of other gram-positive genera.

Cloning and Tn*5* mutagenesis of pCI305. The feasibility of employing the replication probe vectors constructed in this study in the analysis of pCI305 replication genes was tested by cloning *Xba*I-linearized pCI305 in both orientations in *Xba*I-cleaved pCI341, resulting in pCI350 (Fig. 4) and pCI351 (data not shown). Both of these plasmids transformed MG1363Sm protoplasts to Cm^r and replicated in this host without any alteration in copy number as compared with pCI305 based on visual observation of agarose gels. These data also indicated that the *Xba*I site on pCI305 did

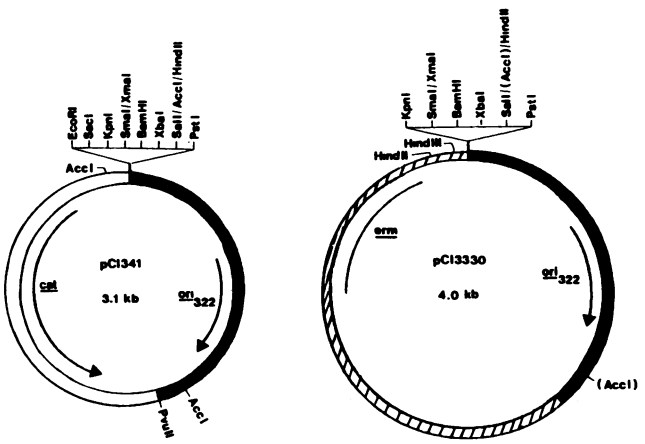

FIG. 3. Restriction maps of replication probe vectors pCI341 and pCI3330. Symbols: ■, pBR322 DNA; □, pC194 DNA; ▨, pAMβ1 DNA. The approximate positions of the pBR322 replication region (ori_{322}) and the *cat* and *erm* determinants of pC194 and pAMβ1, respectively, are shown. The *Acc*I sites on pCI3330 are bracketed to indicate that unmapped *Acc*I sites exist on the pAMβ1 portion of this plasmid.

not reside within a region essential for maintenance of this plasmid.

The position of the replication region on pCI305 was further delineated by subjecting pCI350 to Tn*5* mutagenesis in *E. coli*. Tn*5* inserted preferentially within vector DNA, since 44% of pCI350::Tn*5* derivatives examined had Tn*5* inserted within pCI341 sequences, which formed only 26% of pCI350. However, this did not present substantial problems in isolating pCI350 derivatives with Tn*5* inserted in lactococcal DNA. A total of 20 independent Tn*5* insertions, designated pCI3301 through pCI3320, were mapped in the 8.7-kb pCI305 portion of pCI350 (Fig. 4). A clustering of insertions near the right-hand end of pCI350 was evident, with half of the mapped insertions residing within the 2.6-kb *Xba*I-*Bgl*II fragment in this region.

The ability of the 20 mapped pCI350::Tn*5* derivatives to replicate in *L. lactis* subsp. *lactis* MG1363Sm was examined. All but three of these plasmids readily transformed MG1363Sm protoplasts at frequencies comparable with that of pCI350 and replicated with a copy number similar to that of pCI350 (Fig. 4). However, repeated attempts to transform Tn*5* derivatives pCI3306, pCI3307, and pCI3309 failed, which suggested that in these derivatives Tn*5* had inserted in a region essential for replication of pCI305 in *L. lactis* subsp. *lactis*. These insertions spanned a region of approximately 0.3 kb.

Identification and characterization of the pCI305 minimal replicon. To determine whether the complete pCI305 replication machinery mapped within the rightward 2.6-kb *Xba*I-*Bgl*II fragment of pCI350 (Fig. 4), this fragment was subcloned in pCI341, generating pCI369 (Fig. 4). The insert proved sufficient to support replication in MG1363Sm, indicating that both the origin of replication and any replication gene(s) were within this fragment.

In addition to the possibility of generating phenotypic effects, transposition of Tn*5* results in the introduction of a new set of restriction sites at the insertion point (3); these can be readily used for subcloning regions to the left and right of this point. Among the most useful are *Hpa*I sites situated within the inverted repeats and just 185 base pairs from the termini of Tn*5* (2, 24). These sites were used to generate subclones from pCI3303, pCI3305, pCI3307, and pCI3317, employing either *Xba*I or *Bgl*II as the second cloning enzyme (Fig. 4). Five such subclones were tested for replication in MG1363Sm (Fig. 4). The leftmost boundary of

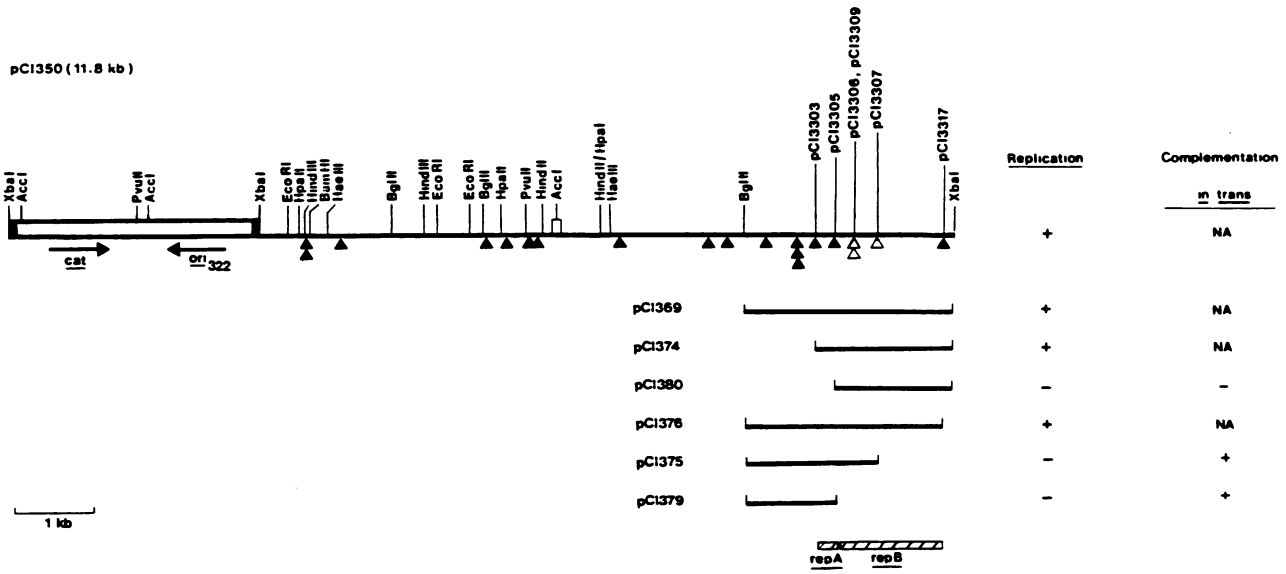

FIG. 4. Cloning, Tn*5* mutagenesis, and subcloning of pCI305. Symbols: □, pCI341 sequences; ——, pCI305 DNA; ■, multiple-cloning site of pCI341; ▲, Tn*5* insertions having no effect on pCI350 replication in *L. lactis* subsp. *lactis*; △, Tn*5* insertions that abolish replication of pCI350 in this host. Subclones generated from individual pCI350::Tn*5* derivatives are drawn below pCI350. Each of the subclones pCI374 through pCI380 contains 185 base pairs of the terminus of Tn*5* at one end. Hybridization analysis with the 2.6-kb *Xba*I-*Bgl*II fragment of pCI350 as a probe confirmed the veracity of these subclones (data not shown). The rightmost column indicates the ability of fragments to sustain replication when provided with the 2.6-kb *Xba*I-*Bgl*II fragment in *trans*. NA, Not applicable. The two domains, *repA* and *repB*, into which the pCI305 minimal replicon can be separated are shown ▨.

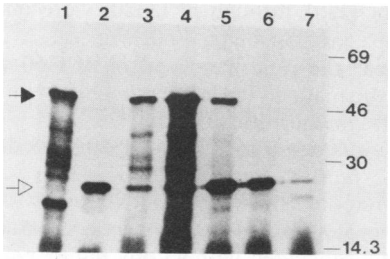

FIG. 5. Identification of a putative replication protein by in vitro transcription-translation studies. DNAs used (lanes): 1, pCI305; 2, pCI341; 3, pCI369; 4, pCI374; 5, pCI380; 6, pCI379; 7, pCI3306. The positions of ^{14}C-labeled molecular weight markers, indicated on the right, are as follows (in decreasing order of size): bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme. The positions of the 26.2-kDa, pC194-derived *cat* gene product (—▷; 23) and the 48-kDa putative replication protein (—▶) are indicated. The Tn*5* *kan* gene product in lane 7 is masked by the *cat* product.

the minimal replicon was defined in pCI374. Deletion of 0.3 kb from this end, as in pCI380, abolished autonomous replication in MG1363Sm. The rightmost limit of the minimal replicon was defined in pCI376. The absence of Tn*5* insertions between the insertion sites in pCI3307 and pCI3317 prevented further elucidation of the rightmost boundary. No copy number effects were noted for these subclones. As determined from these experiments, therefore, the minimal replicon was within a 1.6-kb fragment that extended from the Tn*5* insertion site in pCI3303 to that in pCI3317 (Fig. 4).

Significantly, the Tn*5* insertion in pCI3305 was within the minimal replicon as deduced from subcloning experiments (Fig. 4), which suggested that the minimal region sufficient to direct replication of pCI305 in MG1363Sm was divisible into at least two domains, one mapping between the insertion points in pCI3303 and pCI3305 (*repA*) and the other mapping between the insertion points in pCI3305 and pCI3317 (*repB*). To determine whether either of these regions contained an origin sufficient to drive replication when provided with the complete replication region in *trans*, pCI379, containing *repA* but not *repB*, and pCI380, containing *repB* but not *repA*, were transformed separately into *L. lactis* subsp. *lactis* MG1363Sm containing pCI3331, i.e., the 2.6-kb *Xba*I-*Bgl*II fragment of pCI350 subcloned in the Em^r replication probe vector pCI3330. pCI379 but not pCI380 transformed MG1363Sm(pCI3331) to Cm^r. These results indicated that *repA* was a *cis*-acting element essential for replication but was dependent on the provision of *repB* either in *cis* or in *trans*. Consequently, *repA*, which was at most 0.3 kb in size (Fig. 4), appeared to contain the pCI305 origin of replication, whereas *repB* (1.3 kb) encoded a function(s) that acted in *trans* at or with a product(s) of *repA*.

In vitro transcription-translation studies. Plasmids from a number of overlapping subclones from about the pCI305 replication region were analyzed to determine whether *repB* of pCI305 encodes a protein product(s) that may be involved in replication (Fig. 5). pCI305 produced two major proteins of 20 and 48 kDa (lane 1). Replication-proficient plasmids pCI369 and pCI374 retained production of the 48-kDa product but not of the smaller protein (lanes 3 and 4, respectively). The replication-deficient derivative pCI379 encoded neither the 48-kDa protein nor the 20-kDa protein (lane 6). pCI380, which was incapable of replication in *L. lactis* subsp. *lactis* MG1363Sm but retained *repB* (Fig. 4), still produced the 48-kDa protein but in significantly less amounts (Fig. 5, lane 5). Tn*5* derivatives that abolished replication in MG1363Sm did not produce the 48-kDa protein (lane 7) whereas pCI3305, which contained a Tn*5* insertion within the minimal replicon but remained replication proficient (Fig. 4), did encode this product (data not shown). These results implied that the 48-kDa protein produced by *repB* was involved in the replication of pCI305 in *L. lactis* subsp. *lactis*.

Plasmid stability. The stabilities of pCI369 and pCI3331 in *L. lactis* subsp. *lactis* MG1363Sm were assessed. pCI369 was 100% stable under nonselective conditions for approximately 50 generations. After approximately 100 generations Cm^s derivatives accumulated at a frequency of 1 to 5%. This contrasted with the observation that Em^s segregants of *L. lactis* subsp. *lactis* MG1363Sm(pCI3331) formed >85 and 100% of the population after 50 and 100 generations, respectively.

Host range of pCI305. The host range conferred by pCI305 replication genes was investigated by constructing a plasmid containing the pCI305 replication region but which was devoid of pBR322 sequences as follows. Digestion of pCI369 (Fig. 4) with *Hin*dII (in the multiple-cloning site) and *Pvu*II generated two fragments, the larger of which contained the pCI305 replication functions and the *cat* determinant but not the pBR322 replication region. This fragment was purified and ligated, and *L. lactis* subsp. *lactis* MG1363Sm was transformed to Cm^r. A representative Cm^r isolate contained a 4.2-kb plasmid, pCI378, which yielded the correct restriction patterns (data not shown) and was capable of retransforming MG1363Sm. This plasmid was used to determine that the host range of pCI305 does not extend to *E. coli* HB101 or *B. subtilis* PSL-1 or to other lactic acid bacteria including *Leuconostoc lactis* (A. Harrington, personal communication) and *Lactobacillus casei* (P. J. Cluzel, personal communication), as deduced from failure to transform these hosts. pCI305 differs, therefore, from the only other lactococcal replicons examined, pWV01 (25, 27, 50) and pSH71 (8, 12, 16), in having a narrow host range. Hybridization analysis with a fragment containing the complete replication region of pCI305 as a probe confirmed this nonrelatedness; the probe failed to hybridize to pWV01- and pSH71-based vectors (data not shown).

Construction of pCI305-based shuttle vectors. Stable lactococcus-*E. coli* shuttle vectors based on the pCI305 replication region and possessing a wide range of unique cloning sites were constructed by the following procedure. The 2.6-kb *Xba*I-*Bgl*II fragment of pCI305 that contained the replication region was purified, end filled, and cloned in *Pvu*II-digested pCI341 (Fig. 3), resulting in pCI372 (Fig. 6). A *Bgl*II site was unexpectedly recreated at one junction during this procedure. The number of sites available for cloning purposes was increased by replacing the *Eco*RI-*Pst*I multiple cloning site of pCI372 with a 49-base-pair *Eco*RI-*Pst*I polylinker from pIC20R (33). This resulted in pCI3340, which contained single sites for 10 enzymes, including 6 additional enzymes not present on pCI372 (Fig. 6). Restriction enzyme analysis confirmed the structure of these vectors, which possessed a combined total of 17 unique sites for commonly used enzymes.

DISCUSSION

In this sudy, the identification and preliminary characterization of the minimal replicon of a stable cryptic plasmid pCI305, from *L. lactis* subsp. *lactis* UC317 was described. pCI305 was transformed into a plasmid-free *L. lactis* subsp. *lactis* derivative by using the native Lac Prt plasmid,

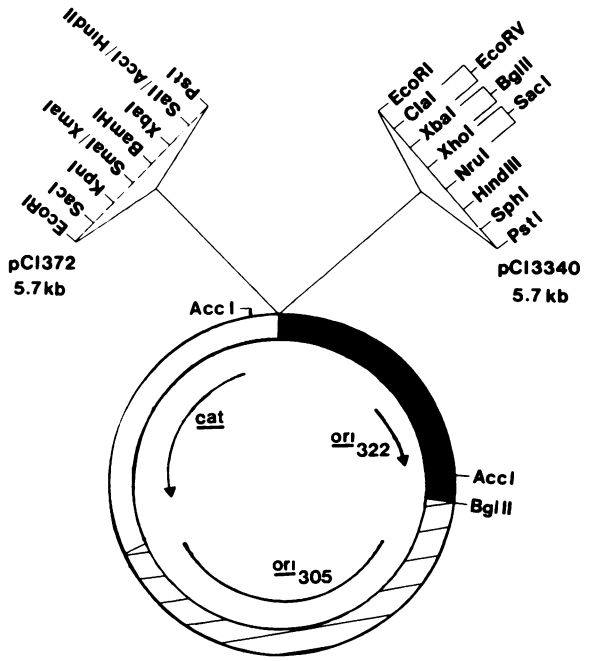

FIG. 6. Restriction maps of shuttle vectors pCI372 and pCI3340. Symbols: ■, pBR322 DNA; □, pC194 DNA; ▨, pCI305 DNA. The approximate positions of the pBR322 (ori_{322}) and pCI305 (ori_{305}) replication regions and the *cat* determinant of pC194 are indicated.

pCI301, of UC317 as a transformation indicator. The high frequency of cryptic plasmid cotransformation, which, despite the large size of pCI301, was comparable to that reported for extraneous antibiotic resistance marker plasmids (14, 20, 41), suggests that this may prove an effective method for the construction of lactococcal strains harboring single, phenotypically cryptic, or nonselectable plasmids and offers a viable alternative to the use of potentially incompatible drug resistance plasmids (20).

The replication region of pCI305 was localized by using Tn*5* mutagenesis. The isolation of a Tn*5* insertion within this region, which had no effect on plasmid replication in *L. lactis* subsp. *lactis*, separated the minimal replicon into two domains. Subcloning of specific overlapping fragments from individual pCI350::Tn*5* chimeras in replication probe vectors confirmed the structure of the minimal replicon as consisting of a 0.3-kb *cis*-acting segment (*repA*) that apparently contained the replication origin and a 1.3-kb region encoding at least one positive, *trans*-acting function (*repB*). This is in agreement with the minimum coding capacity required for the production of the 48-kDa replication-associated protein produced in vitro by *repB*.

The pCI305 minimal replicon was similar to pSH71, the only other well-characterized lactococcal replicon, in being divisible into an origin-containing domain and a region functional in *trans*. However, the plasmids exhibited no detectable homology by Southern hybridization and also differed in the sizes of their respective replication proteins: 48 kDa for pCI305 (Fig. 5) as compared with 27 kDa for pSH71 (W. M. de Vos, personal communication). Furthermore, the minimal replicon of pCI305 displayed a significantly narrower host range than that conferred by both the pSH71 and the related pWV01 replication genes. This narrow host range may reflect a dependence on host-related plasmid replication requirements not found with pSH71-like plasmids. Characterization of plasmids from a greater variety of lactococcal strains will determine the relative frequencies with which these plasmid types occur. In addition, the cloning of replication genes from pCI310, a larger, *L. lactis* subsp. *lactis* UC317-derived proteinase plasmid (22) with which pCI305 is nonhomologous, has been achieved (unpublished data) and, together with continued analysis of the pCI305 minimal replicon, will provide an insight into the factors involved in plasmid replication in multi-plasmid-containing lactococcal strains.

The *trans*-active nature of the *repB* product may be indicative of an indirectly regulated system of plasmid replication, as is the case with other gram-positive replicons, e.g., *S. aureus* plasmids pT181 (36) and pE194 (48) and pUB110 from *B. subtilis* (28). The putative pCI305 replication protein is significantly larger than the positive-acting, diffusible replication proteins of many other gram-positive replicons, which vary in size from 25 to 40 kDa (23, 35, 48). In addition, like pUB110 (28) but unlike pE194 (48), pC194 (23), and the pT181 group of plasmids (39), the available evidence suggests that the pCI305 replication origin does not reside within the replication protein locus. It will also be of considerable interest to determine whether, as in many other small gram-positive plasmids (11, 43), single-stranded intermediates are generated during pCI305 replication.

In addition to behaving as replication probe vectors, pCI341 and pCI3330 (Fig. 3) can be readily converted to *E. coli*–gram-positive genera shuttle plasmids by the insertion of an appropriate replication region. However, the use of pCI341 may be preferable to pCI3330 due to the segregational instability associated with the latter when containing a functional gram-positive replication region and when grown under nonselective conditions. Blunt-ended insertion of a replication-proficient segment of pCI305 into the unique *Pvu*II site of pCI341 proved particularly useful in the construction of stable bridge vectors; the multiple-cloning site remained undisturbed by this procedure. The shuttle vectors produced in this study have proven invaluable in the cloning of lactococcal sequences involved in high-frequency plasmid recombination (unpublished data). The construction of stable cloning vectors may provide an attractive alternative to chromosomal integration as a means of stabilizing heretofore unstable but industrially significant traits in lactococci.

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